Original Research
Pulp Biology

Immunohistochemical Expression of TGF-β1 and Osteonectin in engineered and $Ca(OH)_2$-repaired human pulp tissues

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Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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DOI: 10.1590/1807-3107BOR-2016.vol30.0093

Submitted: Mar 14, 2016
Accepted for publication: Apr 30, 2016
Last revision: June 06, 2016

Abstract: The aim of the present study was to evaluate the expression of transforming growth factor-β1 (TGF-β1) and osteonectin (ON) in pulp-like tissues developed by tissue engineering and to compare it with the expression of these proteins in pulps treated with $Ca(OH)_2$ therapy. Tooth slices were obtained from non-carious human third molars under sterile procedures. The residual periodontal and pulp soft tissues were removed. Empty pulp spaces of the tooth slice were filled with sodium chloride particles (250–425 μm). PLLA solubilized in 5% chloroform was applied over the salt particles. The tooth slice/scaffold (TS/S) set was stored overnight and then rinsed thoroughly to wash out the salt. Scaffolds were previously sterilized with ethanol (100–70°) and washed with phosphate-buffered saline (PBS). TS/S was treated with 10% EDTA and seeded with dental pulp stem cells (DPSC). Then, TS/S was implanted into the dorsum of immunodeficient mice for 28 days. Human third molars previously treated with $Ca(OH)_2$ for 90 days were also evaluated. Samples were prepared and submitted to histological and immunohistochemical (with anti-TGF-β1, 1:100 and anti-ON, 1:350) analyses. After 28 days, TS/S showed morphological characteristics similar to those observed in dental pulp treated with $Ca(OH)_2$. $Ca(OH)_2$-treated pulps showed the usual repaired pulp characteristics. In TS/S, newly formed tissues and pre-dentin was colored, which elucidated the expression of TGF-β1 and ON. Immunohistochemistry staining of $Ca(OH)_2$-treated pulps showed the same expression patterns. The extracellular matrix displayed a fibrillar pattern under both conditions. Regenerative events in the pulp seem to follow a similar pattern of TGF-β1 and ON expression as the repair processes.

Keywords: Dental Pulp; Transforming Growth Factors; Osteonectin; Tissue Engineering.

Introduction

During tooth development, a precise temporal-spatial expression of bioactive glycoprotein-based molecules, known as growth factors (GF), regulates the crosstalk between the epithelial and mesenchymal germ layers,[1,2] leading to odontoblast differentiation.[3] The odontoblasts form a terminally specialized hard tissue. These cells are responsible for secreting the dentin extracellular matrix (DECM) both during the odontogenesis and pulp-dentin complex (PDC) repair.[4,5] After the complete dentin

mineralization, the GF becomes fossilized in the dentin matrix, making the tissue a rich source of bioactive molecules.[6,7]

Because of the secretory activity of odontoblasts, the PDC can react to the external stimuli (caries, trauma) by evoking defense responses.[8] Although moderate carious lesions stimulate the secretory activity of odontoblasts (reactionary dentinogenesis),[4,9] deep cavity preparation or severe carious lesions may lead to the partial destruction of the odontoblastic layer (reparative dentinogenesis). In the reparative dentinogenesis, a population of undifferentiated cells is recruited from the pulp core to the injury site where they differentiate into odontoblast-like cells, starting the deposition of reparative dentin.[10] $Ca(OH)_2$-based materials have been applied in dentistry to preserve the pulp vitality by inducing a dentin bridge formation.[11,12] These materials, because of their high pH, solubilize the DECM, promoting mobilization and recruitment of fossilized GFs.[10,13] Dentin-derived GFs are necessary for the differentiation of dental pulp stem cells (DPSC) into odontoblasts.[14,15] The available data reinforce the hypothesis that the molecular and cellular processes involved in PDC healing and regenerative events recapitulate the odontogenesis.[4,16]

The transforming growth factor-β (TGF-β) family is an important modulator of odontoblast activity, responsible for many molecular events during tooth development and repair.[17] During caries development, the immunoexpression of the isoform 1 (TGF-β1) in odontoblastic cells is enhanced compared with sound teeth.[17] Furthermore, TGF-β1 acts as a potent chemotactic factor for STRO-1-sorted undifferentiated cells.[18] This has been demonstrated by examining the mineralization process coordinated by the dental pulp cells[19] and the effect of the controlled release of TGF-β1 on the pulp cell proliferation and migration. ON is a multifunctional non-collagenous glycoprotein, involved in cell morphogenesis, migration, and differentiation.[20] This protein has been implicated in orchestrating the interactions between cells and its substrates,[21] coordinating cell adhesion, proliferation, and matrix synthesis and turnover.[22]

Because the molecular and cellular processes responsible for dentinogenesis are recapitulated during PDC repair,[4,16] it might be useful to compare the expression of the molecules responsible for both events. The aim of our study was to compare the immunoexpression of TGF-β1 and ON in tooth slice/scaffold (TS/S)-regenerated pulp-like tissue and in $Ca(OH)_2$-repaired pulps.

Methodology

Chemicals

The cell culture medium and reagents were supplied by Invitrogen (Grand Island, NY, USA). All the other reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA), except for phosphate-buffered saline (PBS), which was obtained from Mediatech, Inc. (Herndon, USA), and Poly-L-lactic acid, from Boehringer (Ingelheim, Germany).

Cells

DPSCs, provided by Dr. Songtao Shi were cultured at 37°C in 5% CO_2 in low-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. To conduct the experiments (Figure 1), we used cells from 4^{th}–6^{th} passage.

TS/S preparation

Non-carious human third molars were obtained, after obtaining informed consent and following the approved institutional review board protocol, from young patients (17–23 years old) at the Oral Surgery Clinic (University of Michigan School of Dentistry). The teeth were transversely sectioned at the cervical region. We used a diamond blade at a low speed under cooling with sterile PBS to obtain 1-mm thick tooth slices.[23] The pulp tissue was carefully removed leaving an empty space in the tooth slice. Sodium chloride particles (250–425 μm) were sieved and used to fill the empty pulp chamber, and PLLA solubilized in chloroform (5%) was dropped over the salt particles. TS/S was stored overnight to permit the PLLA polymerization. Subsequently, the salt

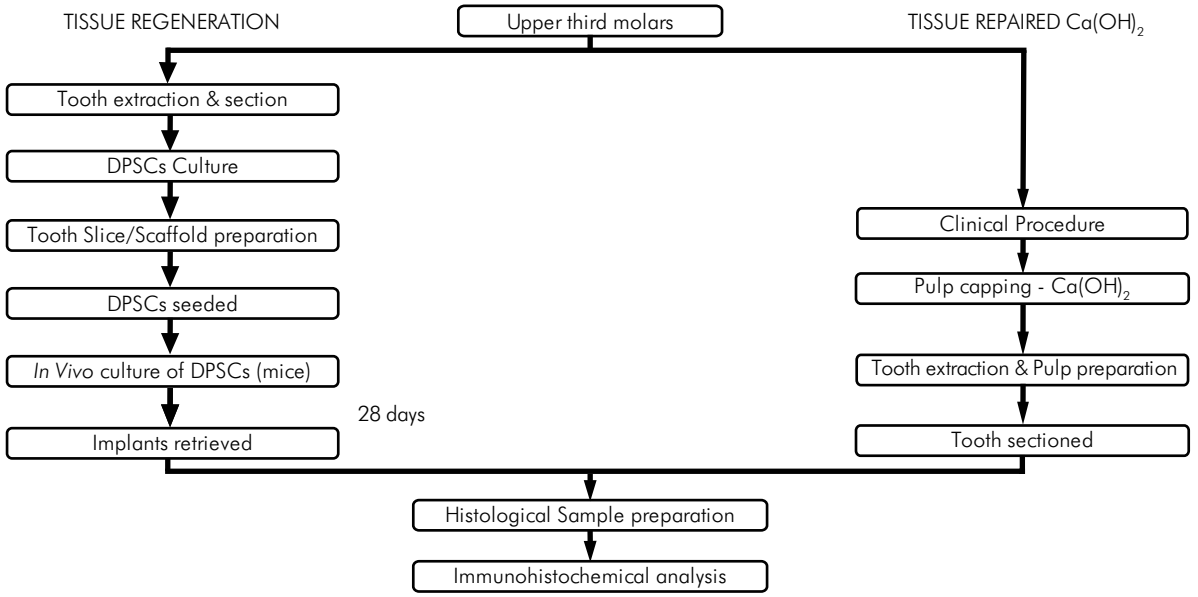

Figure 1. Flowchart of planned protocol with reference to the previous material and method (tooth slice/scaffold and clinical procedure).

was washed out by submersion in distilled water for 24 h (the water was changed 3 times).

Preparation for seeding

Scaffolds were sterilized using ethanol of descending grades (100–70°) and washed with PBS. All scaffolds were treated for 1 min with 10% EDTA (pH = 7.2) and washed again with PBS immediately before cell seeding.

Clinical procedure

As a positive control, we used histological sections provided by Dr. Evandro Piva.[24] Briefly, the human pulp tissue was exposed by a preparation of class I cavities (carbide bur #245) under refrigeration, with cooled PBS. The exposure sites were cleaned using PBS, and the hemorrhage was controlled using sterile cotton pellets. The exposed pulp tissue was capped with calcium hydroxide powder (Biodinâmica, Ibiporã, Brazil). Calcium hydroxide cement (Dycal; Dentsply, Petrópolis, Brazil) was applied over the powder. Cavities were sealed with reinforced zinc oxide–eugenol cement (IRM; Dentsply). Teeth were extracted after 90 days, and the apical root portion was sectioned to improve the formalin penetration inside the pulp.

In Vivo culture of DPSCs

DPSCs, 6×10^5, were re-suspended in a 1:1 DMEM: Growth Factor Reduced Matrigel, seeded in the PLLA TS/Ss or control scaffolds and cultured (at 37°C and 5% CO_2) for 30 min to allow cell attachment. Then, each TS/S was implanted in the dorsum of 5–7-week-old male immunodeficient mouse (CB-17 SCID; Charles River, MA). After 28 days, the implants were retrieved from the dorsum, fixed in 10% buffered formalin at 4°C for 24 h, and demineralized with 10% formic acid at 4°C until the dentin offered no resistance to cutting with a blade (10–15 days). Histological sections (5 mm thick) were prepared from non-carious human third molars and from TS/S as described previously.[14] All experiments in animals were performed following the guidelines reviewed and approved by the University of Michigan Review Board.

Histological sample preparation

The samples were immersed in 5% formic acid until complete decalcification and washed for 48 h in deionized water. The samples were fixed, hemi-sectioned, and inserted in paraffin. Two slices (3 µm) were obtained from each histological

sample (n = 3). Slices were stained with hematoxylin and eosin.

Immunohistochemical analysis

Paraffin blocks were cut into five samples of 3 µm thickness. Each sample was placed on silanized glass (Pró-cite). TGF-β1 antibody (Dakocytomation; Dako, Carpinteria, CA) was used at 1:100 dilution for 18 h at 4°C and ON antibody (1:350; Lab Vision, Fremont, CA), for 18 h at 4°C. We used the streptavidin-biotin method for immunohistochemical analysis. After washing, samples underwent antigenic recuperation treatment with 0.5% pepsin, pH 1.8, for 30 min at 37°C, to reestablish the antigenic sites and break the crosslinking. The slices were washed in tap water, followed by two rinses with distilled water. Unspecific protein blocking was performed by immersion in 10% skim milk solution for 30 min. After the blocking, the slices were washed in water followed by two baths in a Tris-HCl buffer solution. The samples were then incubated with primary antibodies following the manufacturer's instructions and washed in Tris-HCl buffer solution. Incubation with tertiary serum and tertiary complex—Kit LSAB (Dako Corporation, California) — was then performed (both for 30 min). Distilled water and Tris buffer were used to wash the samples. The samples were placed in diaminobenzidine chromogen (AEC, Dakocytomation) for 1 min, counterstained with Mayer hematoxylin for 8 min, and then washed.

Results

Regenerated pulp-like tissues

Twenty-eight days after implantation, DPSCs generated a tissue inside TS/S, with morphological characteristics of a pulp tissue. This tissue, resembling repaired dental pulp, was found at the scaffold filling the pulp chamber (Figures 2 and 3).

Immunohistochemical features of regenerated and repaired tissues

The expression of TGFβ-1 and ON was similar in the repaired and regenerated tissues. In both cases, the ECM staining showed a fibrillar pattern (Figure 2 and 3), reflecting the affinity of TGF-β1 and ON antibodies to the pulp ECM. The pre-dentin from both TS/S and Ca(OH)2-repaired pulp was strongly stained. However, the cellular elements (mainly fibroblasts) were not immunoreactive to the antibodies evaluated here.

Discussion

To analyze the features shared during the repair and regeneration in the PDC, we examined the immunoexpression of TGF-β1 and ON under both conditions.[24] The results showed that the ECM and predentin, both from regenerated and repaired tissues, reacted with TGF-β1 and ON antibodies. During dentinogenesis, a set of GFs orchestrates the epithelial-mesenchymal interactions, leading to odontoblastic differentiation of primitive cells from dental papilla.[25] After DECM mineralization, such GFs are fossilized inside the dentin in their latent form.[26] Thus, dentin becomes a reservoir of latent biomolecules.[25] When solubilizing agents such as $Ca(OH)_2$[10] or EDTA[15] are placed over the dentin walls, the fossilized GFs are released to trigger molecular cascades responsible for repair and regeneration.[6] It is interesting to note that the released GFs are diffused into the pulp tissue to participate in the cell migration and differentiation.[25]

The fibrillar pattern observed in the immunohistochemical analysis is probably due to the presence of collagen and some non-collagen proteins such as fibronectin.[27] Fibronectin mediates the binding of signaling molecules to ECM, playing a critical role during interactions between ECM and the cells.[4] The linking between GFs and ECM seems crucial to cell activation.[6] It is a key event prolonging their action during PDC regeneration and repair.[6] In addition, the activity of GFs is dose-dependent. Thus, an optimal concentration of GFs is needed to trigger a specific biological cascade. GFs might cause cell damage in concentrations higher than a specific dose required.[1] It is possible that the presence of TGF-β1 and ON in the region of predentin and pulp(-like) tissues is due to both the dissolution and activation of the GF, throughout the collagen fibers and some non-collagen proteins such as fibronectin.[28]

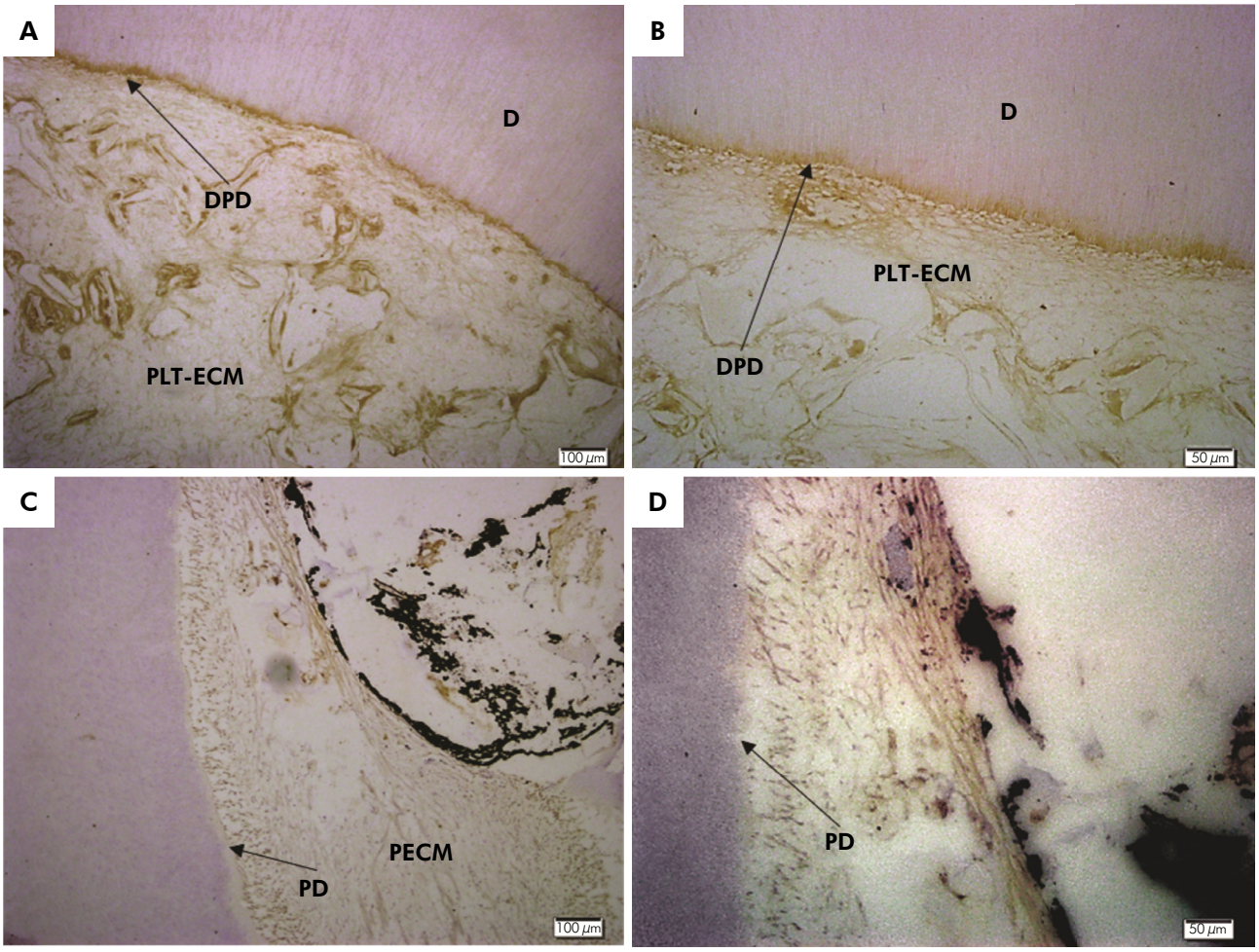

Figure 2. Immunolocalization of ON in regenerated (tooth slice/scaffolds) and repaired ($Ca(OH)_2$) pulps. D: Dentin; P: Pulp; PD: Predentin; CH: $Ca(OH)_2$; TS: Tooth slice; DPD: Demineralized (pre)dentin; PLTECM: Pulp-like tissue extracellular matrix.

The ultimate goal of pulp regeneration strategies is to reconstitute a normal tissue continuum at the pulp-dentin border, regulating tissue-specific processes of secondary and/or tertiary dentinogenesis.[29] Therefore, the newly formed pulp-like tissue must contain odontoblast-like cells capable of secreting predentin in the host organism.[23,29] Here, DPSCs were seeded in TS/S and implanted in the dorsa of immunodeficient mice. TS/S is an effective tool for the investigation of DPSC proliferation and differentiation.[14,15] When the dentin walls of TS/S are treated with EDTA, fossilized GFs are released, and cell differentiation is induced.[30] DPSCs cultured on TS/S under the conditions described here are able to express the three putative odontoblastic markers: dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP-1), and matrix extracellular phosphoglycoprotein (MEPE).

During repair, the stimulus reduces blood flow, inducing the GF (such as TGF-β1) release from the ECM.[10] Samples from healthy human pulps capped with calcium hydroxide for 90 days were also evaluated here. ON was present in the predentin and in the repaired region. Calcium hydroxide can stimulate mineralization, acting like osteodentin, because of its ability to solubilize DECM and release GFs.[10] ON has been found in predentin and in intertubular dentin,[31] which we also observed here. TGF-β1 is incorporated into DECM.[32] After DECM demineralization, TGF-β1 induces stem cell migration to the damaged site and their odontoblastic differentiation.[10] During the ECM formation,[33] TGF-β1

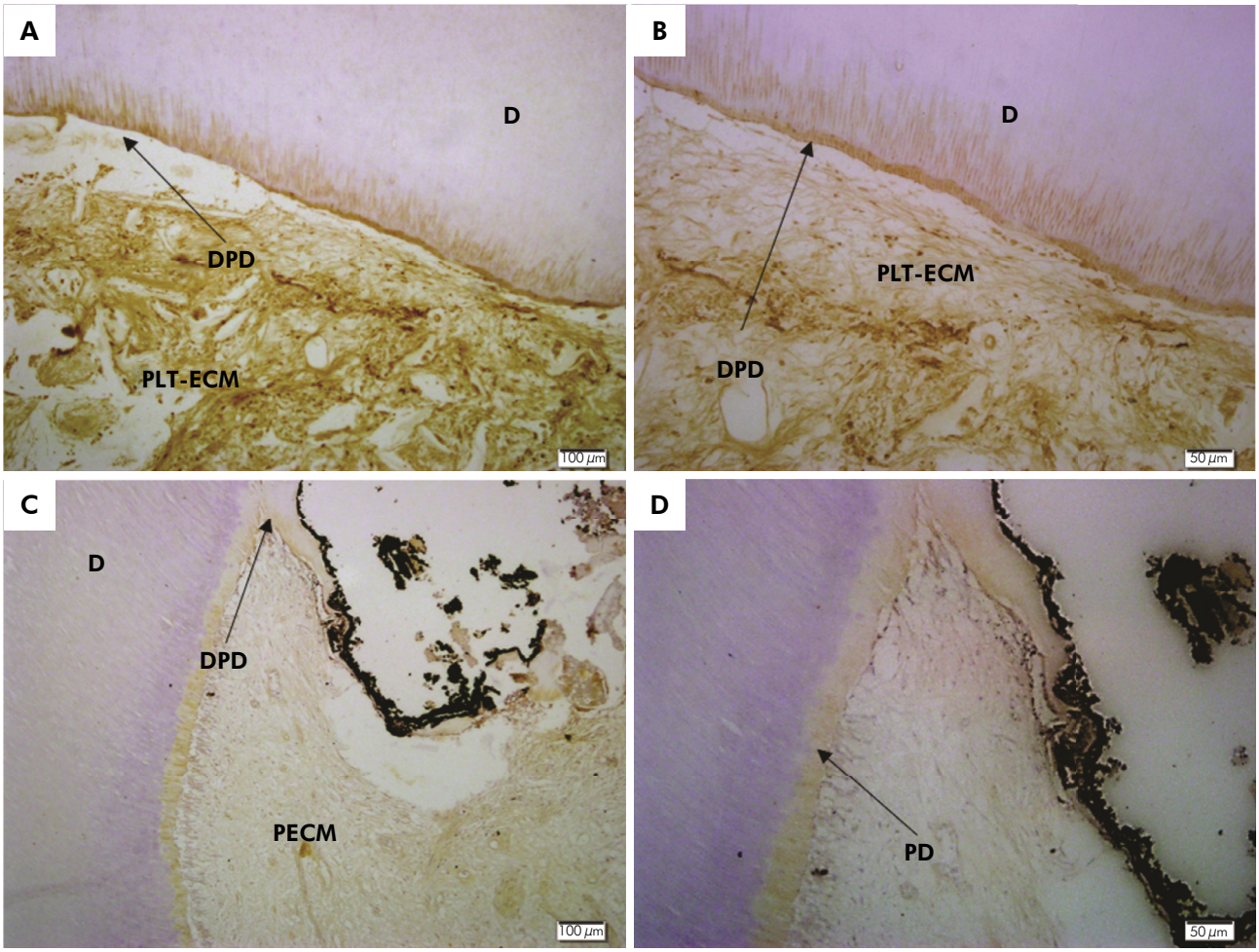

Figure 3. Immunolocalization of TGF-β1 in regenerated (tooth slice/scaffolds) and repaired ($Ca(OH)_2$) pulps. D: Dentin; P: Pulp; PD: Predentin; CH: $Ca(OH)_2$; TS: Tooth slice; DPD: Demineralized predentin; PLTECM: Pulp-like tissue extracellular matrix.

inhibits ECM degradation.[34] It is also an important mediator of ECM remodeling,[36] inducing actin fiber formation.[35] The isoforms of TGF, -β1 and -β3, stimulate the PDC response during the formation of tertiary dentin. The isoform β3 also effectively induces the odontoblastic differentiation during PDC regeneration.[26]

ON is strongly expressed during dentinogenesis.[3] Because of its affinity to hydroxyapatite, ON is involved in molecular cascades determining the hydroxyapatite formation and the crystal stabilization.[22] Thus, this protein plays a central role during reactionary or sclerotic dentinogenesis by modulating the dentin tubule obliteration under pathological conditions.[37] ON is present in the odontoblast layer;[38] its distribution seems to be restricted to the unmineralized predentin of the intertubular dentin and the lamina limitans.[39] When the odontoblast layer is destroyed, ON is released and stimulates the proliferation of a fraction of pulp cells, which differentiate into odontoblasts to form the reparative dentin.[40]

Conclusion

On the basis of our data, we could conclude that the GF evaluated here acts in a similar manner during the pulp repair and regeneration. TGF-β1 is involved in attracting the progenitor pulp cells and the stabilization of ECM, where the stem cells attach. ON is expressed by odontoblast-like cells, indicating their differentiation. The regenerative events in PDC follow a pattern of TGF-β1 and ON expression similar to the pattern seen during the repair process.

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